Stretch- and carbachol-induced ATP release from bladder wall preparations of young and aged mice.

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Abstract:

Aims:

Bladder wall stretch increases tissue tension and releases ATP as part of a transduction process to sense bladder filling. Ageing is associated with bladder fibrosis to produce a stiffer bladder wall: this may augment ATP release and contribute to age-dependent urgency. Muscarinic agonists also release ATP and present a potential target for antimuscarinic agents, but its age-dependency is unknown. This study aimed, in young and old mice, to: i) quantify the relationship between bladder wall stiffness and stretch-dependent ATP release and; ii) characterise muscarinic agonist-dependent release.

Methods

ATP release from young (9-12 weeks) and aged (24 months) mouse bladder wall was measured *in vitro*, with a luciferin-luciferase assay, after stretch or carbachol exposure. Bladder wall stiffness, measured simultaneously during stretch, was compared to histological proportions of connective tissue and detrusor muscle.

Results

With young mice, stretch-activated ATP release required an intact mucosa and was positively associated with wall stiffness. ATP release by carbachol was about four-fold greater compared to stretch. With aged mice: ATP release varied a hundred-fold and no association with stiffness; carbachol release diminished; connective tissue and mucosa thickness increased.

Conclusions

With young mice, stretch or muscarinic agonists potently induce bladder wall ATP release. Stretch-dependent release is proportional to bladder wall stiffness, independent of the extent of stretch. With aged mice dependence of stretch activated ATP release with stiffness was lost. The huge variability of release suggests that aged mice do not form a homogenous cohort and may underlie the heterogeneity in bladder filling sensations.

Introduction

Stretch of the bladder mucosa, or increased pressure across the membrane elicits ATP release that is a sensory mediator to activate nearby afferent nerves.[1] The source of ATP is believed to be the urothelium as shear stress on isolated cells also elicits release[2] and independent of nerve-mediated ATP release. The amount of ATP released increases with the stimulus size,[1,3] thus providing a graded sensory transduction system that matches bladder filling to afferent activation. With several bladder pathologies, such as interstitial cystitis and overactive bladder, ATP release is augmented[4,5] and may contribute to urinary urgency. Muscarinic agonists also release ATP, thus the increase bladder capacity and decrease of urgency by antimuscarinic agents may in part result from reduced ATP release.[3] However, the quantity of ATP release relative to that induced by stretch has not been quantified to indicate if this is a physiologically meaningful stimulus.

The particular physical stressor that initiates ATP release from mucosa/urothelium tissue is less clear. Stretch of any tissue increases its internal, passive tension and either the tissue lengthening itself or the increase of passive tension could be the cause. Most bladder pathologies and ageing are associated with increased extracellular matrix (ECM),[6,7] composed of collagen and elastin fibrils within a ground substance. ECM fibrils are stiffer than detrusor muscle,[8] and excessive ECM deposition (fibrosis) would increase bladder wall stiffness, so that stretch would disproportionately increase passive tension. Thus, if passive tension determines ATP release, fibrosis would increase ATP release.

The elastic (or Young's) modulus, *E*, of any material is a ratio of steady-state tension (stress, normalised to cross-section area), induced by a proportional increase of length (strain). This study investigated if the determinant of ATP release is either the extent of lengthening of bladder wall tissue, or the consequent increase of passive tension. It was carried out in young and old mice when ATP release was measured with a fixed increase of tissue length, with simultaneous

measurement of elastic modulus. Two hypotheses were tested: i) ATP release depends on the elastic modulus (stiffness) of the tissue, with a constant strain change; ii) carbachol-induced ATP release is comparable to that induced by tissue stretch.

Materials and Methods

Animals and ethics. Experiments used tissue from young (9-12 weeks) or old (24 months) C57/BL6 male mice. All animal care and experimental procedures complied with the University Ethics Committee, in accordance with UK legislation under the Animals (Scientific Procedures) Act 1986; Amendment Regulations (SI 2012/3039) and in adherence to ARRIVE guidelines. Animals were procured by the local animal services unit, housed in straw-floored cages at 22°C with a 12hr light-dark cycle and with water and food available *ad libitum*. Animals were killed by a Schedule 1 procedure; cervical dislocation, verified by a lack of corneal and spinal reflexes, and the bladder immediately removed through a laparotomy.

Experimental preparations, contraction protocols and solutions. Excised bladders were pinned to a Sylgard dish with the ventral aspect uppermost, cut along the midline to produce a sheet, the trigone and bladder neck removed, and then bisected. A 1 mm^3 portion, if available, was stored at 4°C in 10% paraformaldehyde for subsequent histology. Longitudinal strips with mucosa (5.0 mm, 1.5-2.0 mm diameter, *intact preparations*) were dissected, tied in a horizontal superfusion trough between a fixed hook and an isometric force transducer (FT20, Hugo Sachs, March-Hugstetten, Germany). With four preparations the mucosa was removed by blunt dissection to generate *denuded preparations*. The transducer was mounted on a micromanipulator that allowed accurate rapid stretch and relaxation of the preparation. The preparation was superfused at 4 $ml.min^{-1}$ with Tyrode's solution (mM): NaCl, 118; KCl, 4.0; $NaHCO_3$, 24; NaH_2PO_4, 0.4; $MgCl_2$, 1.0; $CaCl_2$, 1.8;

glucose, 6.1; Na pyruvate, 5.0; pH 7.4 with 95% O_2/5% CO_2 (37°C). Preparations were equilibrated for 30 min and resting tension adjusted to a final value of 2 mN. To elicit nerve-mediated contractions (abolished by 1 µM tetrodotoxin in a test cohort of six preparations) electrical field stimulation (EFS) was applied *via* Pt-electrodes in the trough walls with 3-s trains of 0.1 ms pulses at frequencies between 1-40 Hz, applied at 90-s intervals. Force-frequency curves from EFS stimulation were used to estimate $T_{max,EFS}$ (maximum tension at high frequency) and $f_{1/2}$ (frequency at $T_{max,EFS}/2$) from the function using KaleidaGraph, Synergy Software, USA:

$$T = (T_{max}.f^n)/(f^n + f_{\frac{1}{2}}^n) \qquad (n \text{ is an empirical constant} = 2)$$

A maximum contractile response to carbachol (10 µM, T_{carb}) was obtained by dilution of an aqueous stock in superfusate. Atropine (1 µM) was also added from an aqueous stock.

ATP measurement and determination of elastic modulus: Stretch and carbachol protocols. The elastic modulus, E, was calculated from changes to tension, ΔT (stress), before and during a maintained rapid stretch (strain) for 100 s. A 1 mm stretch, l, (20% initial length, l_0) was used as a standard intervention throughout, i.e. $l/l_0=0.2$. Thus, $E=\Delta T/(l/l_0)$; units $mN.mm^{-2}$ or kPa. The tension value measured during the rapid stretch was the steady-state value, at the end of the maintained stretch period, and after a period of partial stress-relaxation that occurs in most biological tissues. ATP was measured in 100 µl superfusate samples taken at a fixed point relative to the preparation that minimised mechanical disturbance. Samples were taken before and at 0.1,2,4,6,8,10 and 30 mins after initiation of stretch. At the end of the protocol, samples were taken prior and two minutes after addition of 10 µM carbachol.

Samples were stored on ice before assay with a luciferin-luciferase mix (FLAAM, Sigma-Aldrich, Dorset, UK) and photons measured with a luminometer (Glomax 20/20, Promega). The ATP assay mix was diluted as per manufacturer's instructions. The assay system was calibrated with each

experiment, luminescence was a linear function of ATP (10^{-13}-10^{-6} M) on a log-log plot. The value from a Tyrode's solution sample was subtracted from readings for each experimental sample.

Histology. Fixed specimens were paraffin embedded, sectioned (5 μm), placed on TESPA-coated glass slides and stained with Verhoeff van Gieson (collagen, red; elastin, black; muscle yellow/orange). Muscle and connective tissue (collagen and elastin) areas were measured using colour filters on Image-J (version 2.0.0-rc-43/1.50e) from the whole tissue section.

Data presentation and analyses, experimental design. Data are medians [25,75% interquartiles] as the ATP data sets were not normally distributed; *n*=number of separate preparations. Data sets were compared by ANOVA followed by non-parametric Wilcoxon rank sum tests. Association between variables used Spearman rank order correlation tests (vassarstats.net /corr-rank.html) to obtain a correlation coefficient, *r*, and *p*-value. For stretch-activated ATP release, young-mice data were $\log_{10}$-transformed and a range of mean±3*SD calculated that would encompass 99.7% of data values. This range was used to divide the aged-mice data into three groups: those with values within this range and those either greater or smaller than this range (Fig 3B and accompanying text). The null hypothesis was rejected if $p<0.05$. KaleidaGraph was used for data analysis and curve-fitting with a non-linear iterative fit program. Sample size calculations (*www.3rs-reduction.co.uk*) used previous experimental data with mouse tissue, this suggested group sizes of at least *n*=5-6 for 80% power and a type-I error of 0.05.

Results

Contractile function, mechanical stiffness and histological analysis in intact preparations from young and aged mice. Complete contractile and stiffness measurements were obtained from 18 young

and 21 aged mice (Table 1), histology was available from a subset due to the size of preparations required for ATP release and contraction experiments. Maximum carbachol-induced (T_{carb}) and nerve-mediated ($T_{max,EFS}$) contractions were significantly smaller in aged mice preparations. However, the ratio of the two responses ($T_{carb}/T_{max,EFS}$) was not different, interpreted as no evidence of functional denervation as a cause of reduced force (see Discussion). The frequency required for half-maximum EFS contraction and the atropine-resistant percentage of the EFS (8 Hz) contraction were similar in young and aged mice. Preparations from aged mice were significantly stiffer (Fig 1); median elastic modulus, *E*, was greater although there was wide overlap in the two cohorts. The larger elastic modulus in the aged cohort was consistent with a reduced smooth muscle: connective tissue ratio (Table 1). Increased connective tissue was mainly due to thickening of the mucosa (0.13 [0.11,0.14] *vs* 0.20 [0.18,0.25]mm, young *vs* aged, p=0.001, n=10,10), wherein most collagen was deposited, although there was significant infiltration of the detrusor layer (3.3 [2.8,5.4] *vs* 10.2 [6.4,13.4]% detrusor area, p=0.002, Fig 1B). Bladder dome weights were similar in young and aged mice (25.8±4.0 *vs* 26.2±3.4 mg, n=17,18; p>0.05). Data from mucosa-denuded preparations of four young mice showed contractile responses to carbachol and EFS, and atropine-resistance, were similar to intact preparations, although the elastic modulus in this small cohort was significantly smaller.

Carbachol and stretch-dependent ATP release from young mice intact preparations. ATP release was measured from preparations of 12 young mice. From all there was a small basal ATP release (5 [2,9] fmol.ml^{-1}.mg^{-1}) followed by a larger release with stretch (n=12) or exogenous carbachol (n=10). The profile of ATP release over 30 minutes during and after a 60-s stretch (Fig 2A) peaked in the first two-minutes before declining to the resting level. Stretch-dependent ATP release data are presented as the integrated quantity over ten minutes (hereafter denoted as $\int ATP_{10}$; the shaded region in Fig 2A, Table 1). When stretch-induced ATP had returned to base-line (30 min after

stretch initiation) further ATP measurements were taken before and two minutes after exposure to 10 µM carbachol. For comparison stretch-dependent ATP release after two minutes ($ATP_{2,str}$) was used. Median ATP release was significantly greater with carbachol compared to stretch (Table 1; Fig 2B - note the logarithmic ordinate scale).

The hypothesis was tested that the magnitude of ATP release is a function of tension generated in an intact preparation upon stretch, when the extent of stretch (20% of rest length) was maintained constant in all experiments. For a constant proportional stretch (to 120% of resting length here) the elastic modulus, *E*, is proportional to stretch-dependent tension. Fig 2C shows that $\int ATP_{10}$ indeed showed a significant, positive association with values of *E*, consistent with the above hypothesis (r=0.78, p<0.005, n=12).

In four young mice preparations denuded of mucosa, stretch-dependent ATP release ($\int ATP_{10}$) was very small and similar to basal release (Table 1). Furthermore, with carbachol no increase of ATP above baseline was recorded. Thus stretch- and carbachol-mediated ATP release requires an intact mucosa.

ATP-release and contractile properties of aged mouse intact preparations. Data were obtained from preparations of 20 aged mice; the time course of stretch-mediated release was similar to that of young mouse preparations, but the variability of values among individual preparations was very large, especially within the first two minutes (Fig 3A) and this was reflected in a similar large variability of $\int ATP_{10}$ values (Fig 3B, note the logarithmic ordinate scale). Furthermore, there was no significant relationship (r=0.31, p>0.05, n=19) between $\int ATP_{10}$ and elastic modulus, *E* (Fig 3C). Even within the three subgroups as identified in Fig 3B, as separated by the horizontal lines, there was no relationship

To further compare $\int ATP_{10}$ values between young and aged groups, values were $\log_{10}$-transformed to form more normally distributed sets. The mean and SD of young-mice $\int ATP_{10}$ values were

calculated. The box around the young-mice represent reverse transformed data encompassing the mean and ±3*SD, i.e. if values were greater or smaller than this region there was only 0.3% possibility of this occurring by chance. Extrapolation of the limits of this box showed that $\int ATP_{10}$ values from aged mice could be divided into three groups (Fig 3B): i) those with values greater than those of the young group (*n*=6, high output group; ii) those with $\int ATP_{10}$ values similar to those from young mice (*n*=10, medium output group); and iii) those with very low ATP release values (*n*=4, low output group). This differentiation into three groups of aged mice was also reflected in carbachol-induced ATP release, as well as the basal ATP output before stretch or carbachol interventions (Fig 4A). Thus, in the aged high output group, carbachol also generated large ATP release, along with a high basal release. With the aged medium and low output groups carbachol-induced and basal ATP release was significantly smaller than with young mice. However, this differentiation into ATP release groups was not reflected in contractile responses to EFS and carbachol: between all three groups, contractile function was similar and all significantly smaller than that from young mice (Fig 4B).

Discussion

Stretch-activated ATP release from the bladder wall. These experiments corroborate previous studies that ATP is released from bladder wall preparations subjected to physical strain, or from isolated cells under shear stress,[1-3] and which likely acts as an intermediary to increase afferent activity.[9] An intact mucosa is required, as ATP release is negligible when it was removed. The transduction mechanism whereby physical strain releases ATP remains unclear, but several so-called stretch-activated ion channels are present on urothelial cells, including non-selective cation channels such as Piezo, $TRPV_4$ and epithelial Na^+ (ENaC) channels.[1,10,11] Experiments here with

tissue from young mice showed that strain changes *per se* are not the variable responsible for ATP release but rather the tension induced in the tissue as a consequence of stretch. Thus, ATP release was significantly associated with the tissue elastic modulus, an index of tissue stiffness, with a constant change of strain. The elastic modulus itself is a positive function of strain, when these strains are over ranges near to and above the resting length of the tissue;[12] these experiments used smaller proportional strain changes (20% of resting length) which reflect changes in the intact bladder during filling.[13] A striking feature of the data is the wide range of elastic modulus values in the cohort of young animals, similar to that observed in aged animals.

This dependence of ATP release on tissue stiffness has consequences for pathological conditions where connective tissue replaces smooth muscle,[14] as proteins such as collagen have a high elastic modulus compared to cellular tissue.[15] Thus, a bladder with reducing filling compliance, due to excess connective tissue, will be more sensitive to bladder filling, as wall tension will rise more and so enhance urothelial ATP release. Conversely, generation of a more distensible bladder, as may occur with continued deposition of extracellular matrix and an excess of ground substance,[16] will reduce bladder sensation during filling and result in large bladder volumes with potentially deleterious effects due to raised upper tract pressures. Reduction of excessive deposition of connective tissue will therefore have a double advantage: not only to restore the proportion of detrusor muscle in bladder wall tissue and hence contractile function, but also to recover normal sensation during bladder filling. Several antifibrotic strategies have promising prospects to achieve these goals.[17]

Carbachol-induced ATP release from the bladder wall. The muscarinic agonist carbachol also released ATP from the bladder wall, when the mucosa was intact. Urothelial acetylcholine release itself occurs under conditions similar to those that release ATP, although by different pathways,[3,18] and suggests an intimate relationship between these transmitters. The quantity of ATP released by

carbachol was significantly greater than that elicited by stretch and suggests muscarinic pathways are important regulators of ATP release and could serve as mediators of sensation, independent of stretch-dependent processes. Moreover, a significant site of action for muscarinic receptor antagonists, used to manage the overactive bladder, could be at the mucosa, rather than the detrusor layer with consequential actions to improve filling rather than primarily suppress overactive bladder contractions.

Active and passive properties of mouse bladder wall preparations. Intact detrusor strips from aged mice were less contractile to EFS or carbachol stimulation in agreement with some studies,[19] but at variance with others that reported either no change,[20] or even enhanced contractile function.[21] There was also no evidence for functional detrusor denervation in aged mice as the decline of EFS and agonist-induced responses in aged mice were similar ($T_{carb}/T_{max,EFS}$ similar, Table 1), and the extent of atropine resistance was also the same. However, autonomic dysregulation has been described in aged female mice.[22] The reasons for these different ageing responses in various studies are unclear, but we normalised our data to cross-sectional area of the preparation that included mucosa. Thus, a greater proportion of acontractile mucosa as well as replacement of muscle with connective tissue would both contribute to reduced overall contractile function, accompanied by increased passive stiffness as quantified by a greater elastic modulus. Of interest, the elastic modulus of mucosa is greater than that of the detrusor layer,[23] and as mucosa thickening is a feature of overactive bladder pathologies this bladder wall layer will make a significant contribution to increased bladder wall stiffness in these pathologies. Similar changes to reduced active contractile responses, accompanied by an increased proportion of connective tissue and elastic modulus have also been reported in human detrusor from bladders with contractile dysfunction.[24] We were careful when measuring elastic modulus to always use data from the first stretch protocol, as passive stiffness changes with repeated stretch protocols.[25] A limitation of the study is that it

was not possible to match connective tissue content with either passive stiffness or active contractile performance for every preparation, due to the requirement to dissect two sufficient preparations from each small mouse bladder for active tension and passive tension/ATP release experiments.

Stretch- and carbachol-induced ATP release from the bladder wall of aged mice. A significant relationship between stretch-induced ATP release and tissue elastic modulus was not evident in aged mice, as there was from young counterparts. This was because of the unexpected observation that the magnitude of ATP release varied by almost 100-fold between different animals, from almost none to very much greater amounts than seen in young mice. Overall the median stretch-induced ATP release was not different from that in young mice, in contrast to previous studies,[21,26] but a single value describing the whole data set hid this wide variability. Moreover, the group showing very high stretch-activated ATP release also demonstrated augmented release with carbachol. This was not mirrored in similar variability of contractile performance to EFS and to carbachol (Fig 4). Stretch-activated and carbachol-induced ATP release required an intact mucosa. We can therefore propose this must reflect heterogeneous changes to urothelial function in response to these interventions and has formed the basis of a future study.

The overall implication is that data from aged animals does not represent a homogeneous group and that co-morbid conditions may overwhelm any effect of age *per se*,[20] and if translated to elderly humans would underlie their variable incidence of urinary urgency. One direct hypothesis that may be tested is that when ATP release is much greater in some aged animals or humans, so also should afferent activity as a pathophysiological basis of increased urgency.

Limitations of this study was that a detailed histological survey of each preparation was not possible to allow correlation with the amount of ATP release, due to the amount of tissue needed for

contractile and ATP release experiments. However, enhanced ATP release has been measured with urothelial cells from patients with interstitial cystitis/bladder pain syndrome or animals with induced bladder inflammation.[27] A further limitation of the study was that it was not possible to test directly the absence of ATP release in detrusor-only preparations from the old mice cohort as was done with young mice. The reason for this great variability in old mice therefore deserves more study.

Conclusions: Stretch or an exogenous muscarinic agonist are both potent stimuli to release ATP from the mouse bladder wall; release requires an intact mucosa. With young mice the magnitude of ATP release is a function of bladder wall stiffness; the latter is associated with the extent of connective tissue in the bladder wall. The bladder wall of aged mice is stiffer, but the relation between ATP release and stiffness is absent due to a very large variability of stretch-induced ATP release. Mucosal ATP release is a trigger for afferent nerve activation and it is hypothesised that the large variation of ATP release in aged bladders may in part underlie a similar variation of sensory urgency in humans

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Figures

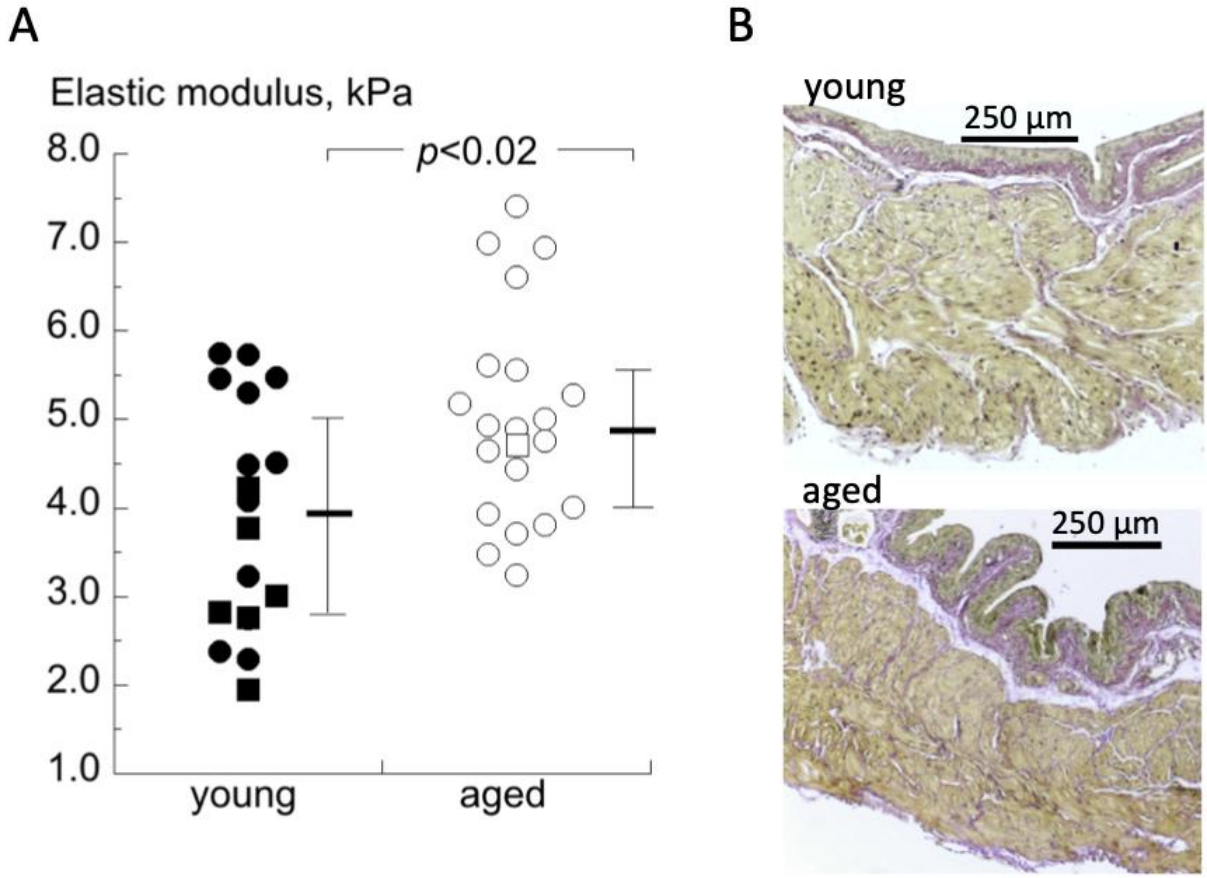

Figure 1. The elastic modulus and histology of intact detrusor preparations. A: Elastic modulus values from young (9-12 weeks, *n*=18) and aged (24 months, *n*=21) mice. Data with accompanying ATP measurements are circles, those without are squares. The error bars show the median and 25,75% interquartiles. Difference between sets tested by a Wilcoxon rank sum test. **B**: Sections of the bladder wall from a young and aged mouse stained with Verhoeff van Gieson preparation

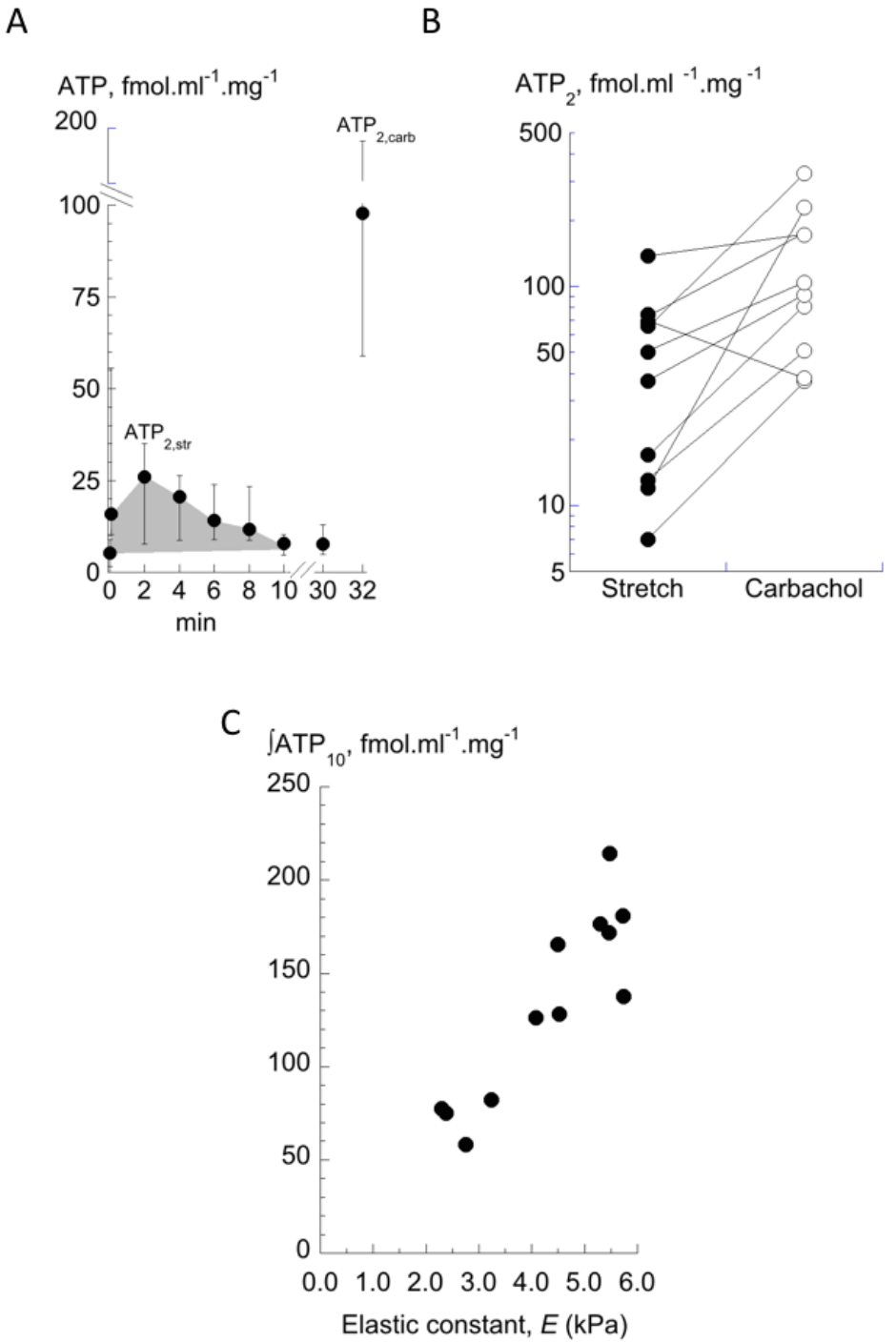

Figure 2. Stretch-activated ATP release in bladder wall preparations from young mice. **A:** Time course of ATP release after the initiation of a 60-s stretch, at *t*=0 min and addition of carbachol at *t*=30 min; median data [25,75% interquartiles]. The shaded area is the integral of ATP release over 10 minutes after initiation of stretch ($\int ATP_{10}$). The data points for $ATP_{2,str}$ and $ATP_{2,carb}$ are shown. **B:** Values of ATP release at two minutes after 60-s stretch ($ATP_{2,str}$) or 60-s exposure to 10 µM carbachol ($ATP_{2,carb}$). The lines connect equivalent values from the same preparation. **C:** The relationship between total ATP release after 60-s stretch ($\int ATP_{10}$) and the elastic modulus, *E*, obtained from the same proportional stretch.

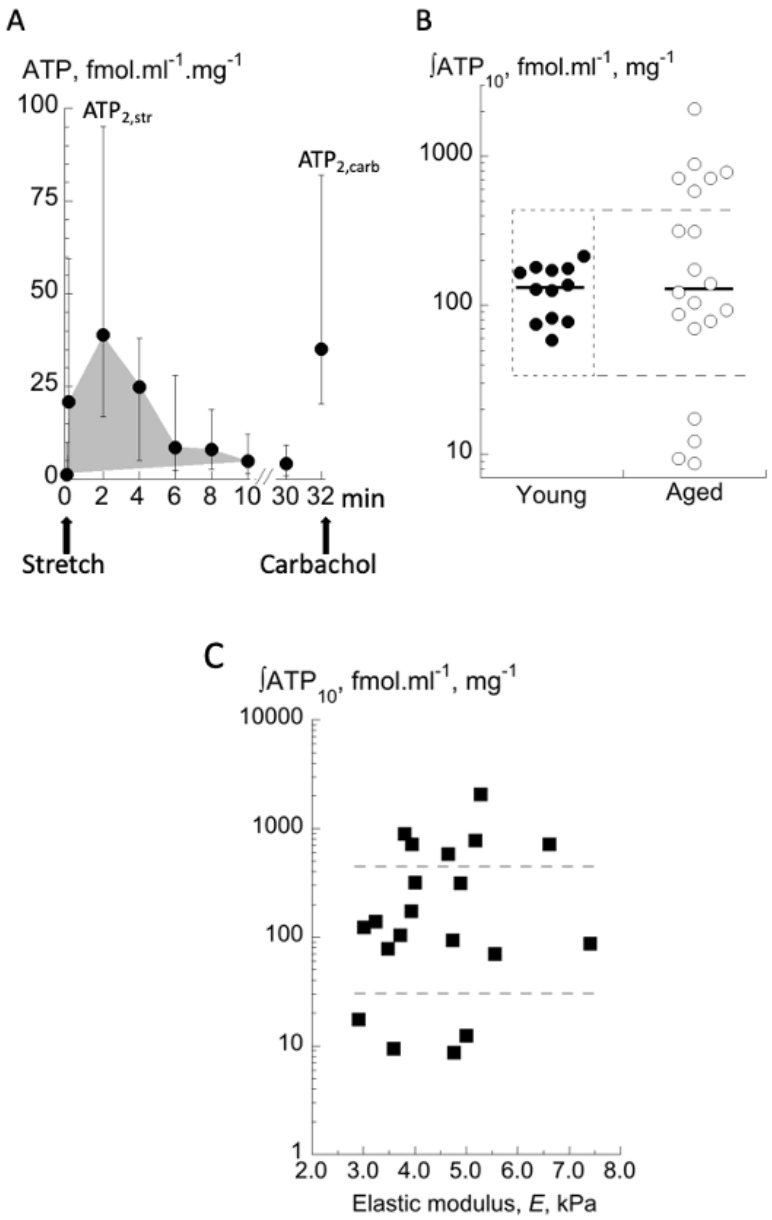

Figure 3. Stretch-activated ATP release in bladder wall preparations from aged mice. A: Time course of ATP release after the initiation of a 60-s stretch at *t*=0 min; median data [25,75% interquartiles] and addition of carbachol at *t*=32 min. The shaded area is $\int ATP_{10,\ str}$; median data [25,75% interquartiles]. The data points for $ATP_{2,str}$ and $ATP_{2,carb}$ are shown. **B:** Values of $\int ATP_{10,\ str}$ of preparations from young and old mice; the dotted-line box for the young cohort outlines the range of data expected for 3*SD above a below the mean of log_{10} transformed data (see text). Extrapolation to the aged-mice data sets lower and upper bounds for the high and low output groups. Horizontal bars show median values of data sets. **C**: $\int ATP_{10}$ values as a function of elastic modulus, *E*, obtained from the same proportional stretch. The horizontal lines separate the data points into the same three sets as in part B.

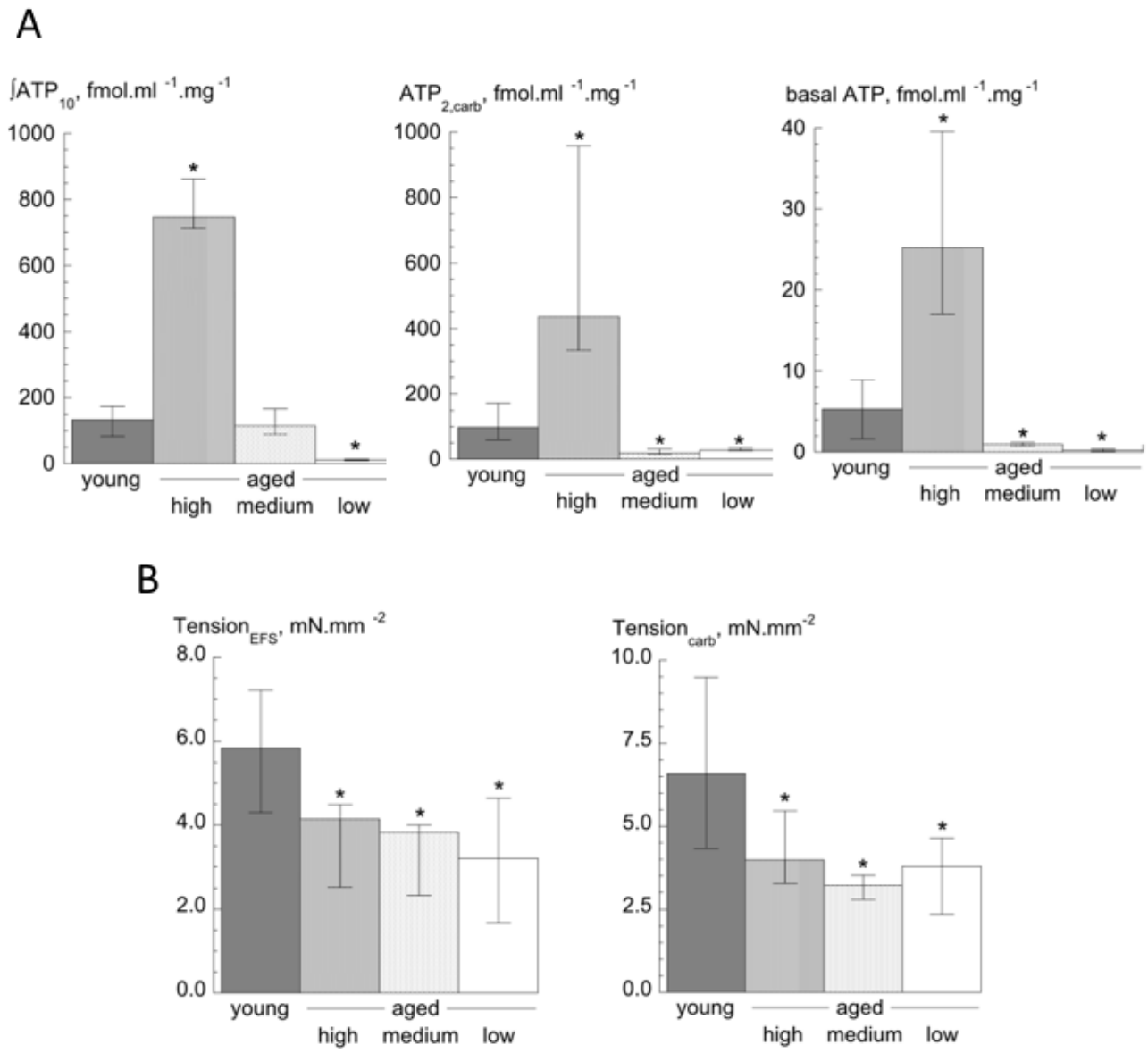

Figure 4. Comparison of ATP release and active contractile properties of young and aged mice. A: Values of $\int ATP_{10}$, $ATP_{2,\ carb}$ and basal ATP release for preparations from young (*n*=12) and aged (*n*=20) mice. The aged mice data are divided into the three stretch-dependent ATP release cohorts: high output (*n*=6); medium output (*n*=10); and low output (*n*=4). **B:** Contractions to electrical field stimulation (EFS, 8Hz) and carbachol (10 µM), data from the young mouse cohort and three aged mice cohorts, *n*-values as in part A. **p*<0.05 *vs* young mice data.

Table 1. Contractile variables and passive stiffness of intact preparations from young (9-12 weeks) and old (24 month) mice. Median [25%, 75% interquartiles] data. *p<0.05 vs intact young mice, #p<0.01 $\int ATP_2$ carbachol *vs* $\int ATP_2$ stretch; Wilcoxon unpaired tests; n = number of preparations.

T_{carb}, peak tension with 10 µM carbachol; $T_{EFS,max}$, maximum estimated tension with electrical field stimulation (EFS); $f_{1/2}$, EFS frequency to generate $T_{max/2}$; SM:CT ratio = smooth muscle:connective tissue ratio;$\int ATP_{10}$, integral of ATP release over 10 minutes from beginning of stretch; $ATP_{2,str}$, ATP release at two minutes from beginning of stretch: $ATP_{2,carb}$, ATP release at two minutes after exposure to carbachol.

	Young mice (intact)	Young mice (denuded)	Aged mice (intact)
T_{carb}; mN.mm^{-2}	6.58 [4.32, 9.48] (18)	8.91 [8.08,11.42] (4)	3.99 [3.12, 6.05]* (21)
$T_{EFS,max}$; mN.mm^{-2}	5.84 [4.30, 7.22] (18)	6.31 [4.76, 7.54] (4)	3.91 [2.04, 6.31]* (21)
T_{carb}/T_{EFS}	1.06 [0.80, 1.29] (18)	1.12 [0.81, 1.57] (4)	1.01 [0.81, 1.86] (21)
$f_{1/2}$, Hz	7.7 [6.4, 9.3] (18)	7.7 [7.0, 7.8] (4)	8.0 [6.4, 10.1] (21)
Atropine resistance, %	72.7 [61.9, 77.2] (16)	57.7 [50.7, 60.9] (4)	63.8 [49.3, 71.2] (21)
Elastic modulus; kPa	3.94 [2.80, 5.10] (18)	1.78 [1.68, 2.26]* (4)	4.76 [3.93, 5.56]* (21)
SM:CT ratio	5.32 [3.33, 6.38] (10)		1.86 [1.59, 2.57]** (10)
$\int ATP_{10\ str}$, fmol.ml^{-1}.mg^{-1}	128 [81, 173] (12)	9.6 [8.1, 12.8]* (4)	131 [77, 618] (20)
$ATP_{2\ str}$, fmol.ml^{-1}.mg^{-1}	27 [12, 67] (12)	8 [5, 12]* (4)	41 [12, 280] (20)
$ATP_{2\ carb}$, fmol.ml^{-1}.mg^{-1}	97 [59, 172]# (10)	0.0 [0.0, 0.0]* (4)	31 [22, 78] (19)